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Possible Roles of Sulfur-Containing Amino Acids in a Chemoautotrophic Bacterium-Mollusc Symbiosis

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Abstract. Invertebrate hosts of chemoautotrophic symbionts face the unique challenge of supplying their symbionts with hydrogen sulfide while avoiding its toxic effects. The sulfur-containing free amino acids taurine and thiotaurine may function in sulfide detoxification by serving as sulfur storage compounds or as transport compounds between symbiont and host. After sulfide exposure, both taurine and thiotaurine levels increased in the gill tissues of the symbiotic coastal bivalve *Solemya velum*. Inhibition of prokaryotic metabolism with chloramphenicol, inhibition of eukaryotic metabolism with cycloheximide, and inhibition of ammonia assimilation with methionine sulfoximine reduced levels of sulfur-containing amino acids. Chloramphenicol treatment inhibited the removal of sulfide from the medium. In the absence of metabolic inhibitors, estimated rates of sulfide incorporation into taurine and thiotaurine accounted for nearly half of the sulfide removed from the medium. In contrast, amino acid levels in the nonsymbiotic, sulfide-tolerant molluscs *Geukensia demissa* and *Yoldia limatula* did not change after sulfide exposure. These findings suggest that sulfur-containing amino acids function in sulfide detoxification in symbiotic invertebrates, and that this process depends upon ammonia assimilation and symbiont metabolic capabilities.

Introduction

Aquatic habitats such as deep-sea hydrothermal vents, mangrove swamps, eelgrass beds, and sewage outfall sites tend to be characterized by high levels of the metabolic toxin hydrogen sulfide (Fenchel and Riedl, 1970; Felbeck *et al.*, 1981; Cavanaugh, 1983). Hydrogen sulfide diffuses freely across respiratory surfaces and therefore cannot be excluded from tissues (Denis and Reed, 1927; Julian and Arp, 1992). It also reversibly inhibits cytochrome *c* oxidase (Lovatt Evans, 1967; Nicholls, 1975) and decreases hemoglobin oxygen affinity (Carrico *et al.*, 1978). Consequently, animals living in these environments require physiological mechanisms to cope with hydrogen sulfide toxicity.

Invertebrates that harbor symbiotic chemoautotrophic bacteria also require sulfide, for their symbionts utilize the chemical energy generated by hydrogen sulfide oxidation to fix carbon dioxide into carbohydrates (Felbeck *et al.*, 1981; Ruby *et al.*, 1981; Cavanaugh, 1983). The invertebrate host delivers hydrogen sulfide and oxygen to its symbionts and relies upon the symbiont-produced carbohydrates as a source of nutrition (Rau, 1981; Southward *et al.*, 1981; Felbeck, 1985). This type of symbiotic relationship exists in over 100 species from at least five invertebrate phyla (Cavanaugh, 1994).

Sulfide detoxification and transport strategies have been well studied in the coastal protobranch bivalves of the genus *Solemya*, which harbor approximately 1×10^9 intracellular chemoautotrophic symbionts per gram wet weight of gill tissue (Cavanaugh, 1983; Felbeck, 1983). These clams generally form U- or Y-shaped burrows in shallow-water reducing sediments and pump oxygenated water through their burrows (Frey, 1968), which enables them to simultaneously acquire the dissolved oxygen and sulfide needed for chemoautotrophy.

To detoxify sulfide, *Solemya velum* and *S. reidi*, two well-studied species, use several strategies apart from utilization by their symbionts. For example, *S. velum* has two types of cytoplasmic hemoglobins: one binds oxygen, and a second, which combines with sulfide to form ferric hemo-

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Abbreviations: MSX, methionine sulfoximine; ASW, artificial seawater; FIA, flow injection analysis; POS, polarographic oxygen sensor.

globin sulfide, may mediate sulfide transport to the symbionts (Doeller *et al.*, 1988). The sulfide-binding form of cytoplasmic hemoglobin is not present in *S. reidi* (Kraus *et al.*, 1992); rather, sulfide oxidation occurs in the mitochondria, hematin, and sulfide-oxidizing bodies (Powell and Somero, 1985, 1986; Powell and Arp, 1989).

Synthesis of sulfur-containing free amino acids may be an additional strategy for sulfide detoxification and transport in chemoautotrophic symbioses (Alberic and Boulegue, 1990; Conway and McDowell Capuzzo, 1992; Pranal *et al.*, 1995; Pruski *et al.*, 1997). In many marine organisms, including solemyid clams and some deep-sea symbiotic species, taurine (2-aminoethanesulfonic acid) is the dominant free amino acid (Alberic and Boulegue, 1990; Conway *et al.*, 1992; Conway and McDowell Capuzzo, 1992; Pranal *et al.*, 1995; Lee *et al.*, 1997; Pruski *et al.*, 1997). Intracellular free amino acid pools function in cell volume regulation (Pierce, 1982; Yancey *et al.*, 1982; Rice and Stephens, 1988), and the primary function of taurine may be as a compatible osmolyte, a function which is conserved throughout the animal kingdom (Allen and Garrett, 1971; Huxtable, 1992).

The exceptionally high levels of taurine and the related amino acid thiotaurine (2-aminoethanethiosulfonic acid) in several chemoautotrophic symbioses have prompted investigators to consider additional roles for these amino acids. For example, taurine appears to be an end product of ammonia assimilation in *S. reidi* (Lee *et al.*, 1997), and may be involved in sulfur cycling in *S. velum* (Conway and McDowell Capuzzo, 1992). Similarly, thiotaurine may function in sulfur cycling in deep-sea symbiotic bivalves (Alberic and Boulegue, 1990; Pruski *et al.*, 2000; Pruski, 2001). Taurine and thiotaurine may serve as important sulfide storage compounds, allowing *S. velum* to maintain low levels of intracellular sulfide. Under conditions where sulfide is low or absent, taurine and thiotaurine may provide sulfide to mitochondrial and symbiont sulfide oxidation pathways. Several species of aerobic gram-negative soil and enteric bacteria use taurine as a sulfur, carbon, and nitrogen source (Stapley and Starkey, 1970; Smiley and Wilkinson, 1983; Seitz *et al.*, 1993; King and Quinn, 1997; Chien *et al.*, 1999; Cook *et al.*, 1999; Reichenbecher and Murrell, 1999), and similar metabolic pathways may be present in the gram-negative chemoautotrophic symbionts. Additionally, taurine and thiotaurine production could facilitate sulfide detoxification under anaerobic conditions, thereby preventing deleterious effects such as the reaction of sulfide with metalloproteins. This strategy would be particularly beneficial in the burrow environment of solemyid clams, in which oxygen and sulfide levels vary.

In the present study, we tested the hypothesis that taurine and thiotaurine levels in intact gills of *S. velum* increase upon exposure to sulfide, which would occur if these amino acids are involved in sulfur storage or cycling. The effects of sulfide exposure on levels of hypotaurine (2-aminoethanesulfinic acid), a possible precursor to taurine and thiotaurine, were also examined. Host- and symbiont-specific metabolic inhibitors were used to distinguish the roles of the *S. velum* host and its symbionts in ammonia assimilation and the maintenance of taurine, thiotaurine, and hypotaurine pools. Flow-through respirometry studies were conducted with *S. velum* to quantify rates of ammonia flux and to determine whether sulfide consumption is related to fluctuations in amino acid pools. In parallel studies, we tested for correlations between sulfide and free amino acid levels in two sulfide-tolerant, nonsymbiotic bivalve species: the estuarine mussel *Geukensia demissa* and the protobranch bivalve *Yoldia limatula*.

Materials and Methods

Specimen collection and maintenance

Solemya velum Say, 1822, *Geukensia demissa* (Dillwyn, 1817), and *Yoldia limatula* (Say, 1831) were collected by the Marine Resources Center of the Marine Biological Laboratory (Woods Hole, MA) and shipped on the day of collection. In our laboratory the clams were maintained for up to 5 days in a flow-through respirometry system (see http://www.wsu.edu/~rlee/respirometer/respirometer.htm and Fig. 1). In each experiment, the temperature of the respirometry system was regulated to match the ambient water temperature at which the clams were collected, which ranged seasonally from 5 to 15 °C over a nearly 2-year period. These temperature differences did not affect the free amino acid composition. Consumption of sulfide, oxygen, and ammonia was measured in a subset of experiments conducted in the late spring and at 15 °C. *Solemya velum* and *Y. limatula* were maintained in 0.45-μm filtered, 35‰ artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) supplemented with 50 μM ammonia. *Geukensia demissa* was maintained in filtered, 30‰ ASW supplemented with 50 μM ammonia. The ASW solution in the chambers was kept mixed with magnetic stir bars (60 to 100 rpm). ASW flow rates ranged from 0.18 to 0.19 ml min^{-1}.

Maintaining the clams in a flow-through respirometry system allowed for constant monitoring of experimental conditions. The oxygen concentrations in the chamber outflows were determined with a polarographic O_2 sensor (POS), whereas the concentrations of sulfide and, in some experiments, ammonia were measured by flow injection analysis (FIA). Throughout this paper, sulfide refers to ΣH_2S (primarily the sum of HS^- and H_2S), and ammonia refers to ΣNH_3 (the sum of NH_3 and NH_4^+). Outflows were pumped directly through a flow cell containing a sulfide-insensitive gold microcathode POS (Orbisphere 2120, Geneva) followed by a series of FIA injection sample loops

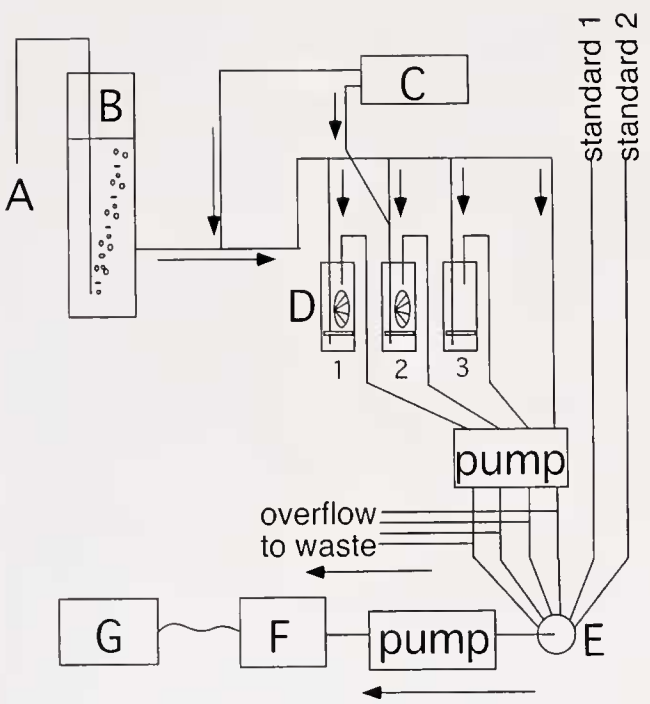

Figure 1. Schematic of flow-through respirometry system. (A) CO_2, N_2, and O_2 gas mixture. (B) Seawater-gas equilibration column with pH regulation. (C) Syringe pump for metering sodium sulfide stocks into seawater entering the chambers. The syringe pump is also used to meter inhibitor into chamber 2. (D) Water-jacketed chambers containing clams, with at least one empty chamber to function as a control. Outflows from the chambers are pumped to a pneumatically actuated six-way stream-selector valve (E). The position of this valve determines whether the outflow goes to waste or to the analysis system. The analysis system consists of a flow injection analyzer for sulfide and ammonia determination and an O_2 sensor mounted in a flow cell (F). The analyzer and stream-selector valve are linked to a data acquisition and automated control system (G).

(Rheodyne Type 50 valves with Rheodyne 5701 pneumatic actuators, Rohnert Park, CA).

The FIA determination of sulfide involved derivitizing the sample stream with 1.5 m*M* 2,2′-dithiodipyridine, which forms a stable product with an absorbance maximum at 343 nm (Svenson, 1980). The derivitized sample was loaded into an injection loop and sent to a variable wavelength detector (Hyperquan VUV-20, Colorado Springs, CO). This method produces 343-nm absorbance peaks proportional to sulfide concentration (Svenson, 1980). Ammonia was determined by a modification of the FIA protocol of Willason *et al.* (1986). The sample was loaded into an injection loop and then treated with NaOH to raise the pH, converting ammonium to ammonia gas. Ammonia passes through a Teflon membrane into a carrier stream containing phenol red, raising the pH of the phenol red solution. The resulting color change (absorbance at 560 nm) is monitored with a detector fabricated from a green LED and a phototransistor. POS and FIA detector outputs were continuously recorded with a data acquisition system (Sable Systems, Datacan V, Henderson, NV), which was also used to control FIA injection valve actuation.

Three respirometer chambers containing clams, one control chamber, and two standards were connected to a six-way stream-selector valve (Fig. 1). Every 0.13 h the valve was automatically switched, allowing sampling from all six channels every 0.8 h. Peak height (for FIA) or average output (for POS) was calculated to determine concentration differences between chambers containing clams and the control chamber. These data were continuously monitored to ensure, in the case of oxygen measurements, that the clams were not exposed to hypoxic conditions. Direct measurements of sulfide in samples taken from the respirometer outflow were used to standardize sulfide data obtained by FIA. Fluxes were calculated as follows: μmol sulfide $\cdot$ g^{-1} wet weight $\cdot$ h^{-1} = (concentration$_{\text{experimental chamber}}$ − concentration$_{\text{control chamber}}$) × (flow rate in l $\cdot$ h^{-1}) × (g^{-1} wet weight).

Experimental treatments

In all experiments, the clams were acclimated for 24 h in ASW prior to sulfide or inhibitor exposure. In experiments in which clams were exposed to sulfide, a syringe pump (Harvard Apparatus 944, South Natick, MA) was used to meter a 20 m*M* Na_2S solution (Fig. 1C) into the ASW solution before its entry into the respirometer chambers. Final sulfide concentration in the chambers was 0.45 ± 0.05 m*M*. Clams assayed for amino acid levels were exposed to sulfide for 24 h. In some experiments, a metabolic inhibitor was added to the ASW solution after the 24-h acclimation period. The final concentrations of these inhibitors were 0.9 m*M* chloramphenicol (Sigma, St. Louis, MO), 0.02 m*M* cycloheximide (Sigma), and 0.25 m*M* methionine sulfoximine (MSX; Sigma). In experiments in which sulfide consumption was examined, the clams were acclimated for 24 h in ASW, then exposed to sulfide for up to 100 h. During the final 24 h of the sulfide exposure, metabolic inhibitors were added to the ASW solution. The clams were weighed before and after treatment.

Amino acid analyses

After experimental treatment, the bivalves were opened by severing their adductor muscles. The gills and foot of each clam were dissected free of other tissues, blotted briefly on a paper towel, then individually frozen in liquid nitrogen and stored at −80 °C. The gills and feet were individually homogenized in distilled water (1:25, tissue/dH_2O) on ice. To precipitate proteins, the homogenates were treated with 5% sulfosalicylic acid (Sigma) in a 1:10 ratio of sulfosalicylic acid/homogenate (Lee and Slocum, 1988). The solutions were centrifuged for 5 min at 16,000 × *g*, and the supernatants were stored at −80 °C.

Total free amino acids were quantified in a Beckman

6300 amino acid analyzer following a protocol modified from Lee and Slocum (1988). The samples were diluted (1:30, by volume) with Li-S buffer (96.8% H_2O, 1% LiCl, 1% thiodiglycol, 0.7% HCl, 0.5% benzoic acid; pH 2.2; Beckman Coulter, Inc., Fullerton, CA). Of the 240–250 μl of sample or standard loaded onto the sample loops, 50 μl of each solution was analyzed. The amino acids were separated in Li-A buffer (98% H_2O, 1% Li citrate, 0.5% LiCl, 0.5% HCl; pH 2.8; Beckman Coulter) on a 10-cm ion exchange column and reacted in-line with ninhydrin solution. Absorbances were monitored at 570 nm and 440 nm. After preliminary experiments, only taurine, hypotaurine, and thiotaurine were quantified. The standards were 200 μM taurine (Sigma) and 20 μM hypotaurine (Sigma) in Li-S buffer. The thiotaurine standard was prepared by dissolving 0.0011 g hypotaurine and 0.05 g $Na_2S \cdot 9H_2O$ (Fisher Scientific, Fair Lawn, NJ) in deionized water, heating to 100 °C, acidifying the solution with 1 *M* HCl, and evaporating the solution (Cavallini *et al.,* 1963). This standard was verified by mass spectrometry.

The temperature of the column affected the separation of thiotaurine from taurine and the reactivity of hypotaurine with ninhydrin. Thiotaurine could be detected only at 70 °C. The values of hypotaurine reported here are only from analyses run at 45 °C, because hypotaurine was not as reactive with ninhydrin at 70 °C. The temperature did not affect taurine levels. Reported values for taurine are averages between levels detected at 45 °C and 70 °C.

Results are presented as the mean ± the standard error and as average rates of synthesis per gram wet weight over a 24-h period [(amino acid level in g^{-1} wet weight in clams exposed to sulfide) − (amino acid level in clams not exposed to sulfide)/24 h] assuming the synthesis rate was linear over the 24-h period. Differences among means were detected using one-way ANOVA for each amino acid in *S. velum* samples (Statistica, Statsoft, Inc., Tulsa, OK). The appropriate comparisons were analyzed with Fisher's LSD procedure (Statistica). The *G. demissa* and *Y. limatula* amino acid data were analyzed with two-sample *t* tests (Statistica).

Results

Sulfide exposure increased taurine and thiotaurine levels in S. velum *but not in two nonsymbiotic bivalves*

Specimens of *Solemya velum* exposed to sulfide had significantly more taurine ($P = 0.0034$) and thiotaurine ($P < 0.0001$) in their gill tissue than clams not so exposed (Table 1). These values equate to average rates of change in taurine and thiotaurine levels of 0.89 μmol $\cdot$ g^{-1} wet weight $\cdot$ h^{-1} and 0.22 μmol $\cdot$ g^{-1} $\cdot$ h^{-1} over the 24-h incubation period. Hypotaurine levels were not significantly affected ($P = 0.064$). Cysteine and methionine, two other sulfur-containing amino acids, were below the limits of detection in all samples. Levels of the most abundant non-sulfur-containing free amino acids—alanine, glutamate, and aspartate—were unaffected by sulfide exposure (data not shown). In preliminary experiments with sulfide-exposed clams, free amino acid profiles of *S. velum* foot (symbiont-free) and gill tissues were similar (data not shown), as observed previously (Conway and McDowell Capuzzo, 1992). In subsequent experiments with metabolic inhibitors, only taurine, hypotaurine, and thiotaurine levels in the symbiont-containing gill tissues of *S. velum* were quantified.

Gill tissue from the nonsymbiotic bivalve species *Geukensia demissa* and *Yoldia limatula* contained less taurine ($P < 0.0001$, non-sulfide-exposed) than *S. velum* (Table 1), but comparable levels of hypotaurine ($P = 0.068$) and thiotaurine ($P = 0.648$). The concentrations of these amino acids were the same whether or not the bivalves were exposed to sulfide (all comparisons, $P > 0.05$).

Metabolic inhibitors decreased taurine and thiotaurine levels in S. velum *gills*

To investigate the role of the chemoautotrophic symbionts, clams were exposed to chloramphenicol, a specific

Table 1

Effects of sulfide exposure and inhibitors on taurine, hypotaurine, and thiotaurine levels in gills of three bivalve species

		Taurine		Hypotaurine		Thiotaurine	
Species	Treatment (*n*)	−Sulfide	+Sulfide	−Sulfide	+Sulfide	−Sulfide	+Sulfide
Solemya velum	No inhibitor (16)	100.3 ± 5.5[a]	120 ± 5.4[a,b]	13.7 ± 1.4	11.4 ± 1.3	0.35 ± 0.2[a]	5.4 ± 0.6[a,b]
	Chloramphenicol (9)	96.5 ± 6.5	102.2 ± 4.5[b]	12 ± 0.8	12.9 ± 1.4	0.42 ± 0.2[a]	3.6 ± 0.8[a,b]
	Cycloheximide (5)	100.1 ± 6.6	109.5 ± 3.9	20.3 ± 2.6	15.1 ± 2.2	1.4 ± 0.2[a]	5.5 ± 0.7[a]
	MSX (7)	91.7 ± 10	96.1 ± 5.7[b]	18.5 ± 3	12.7 ± 4	0.79 ± 0.3[a]	6.5 ± 0.6[a]
Geukensia demissa	No inhibitor (7)	37.9 ± 1.9	39.9 ± 1.2	4.7 ± 0.7	4.5 ± 0.6	0.65 ± 0.2	0.95 ± 0.4
Yoldia limatula	No inhibitor (3)	62 ± 5.5	56 ± 6.7	10.9 ± 1.5	9.9 ± 1.3	0.16 ± 0.16	0.24 ± 0.03

Data are mean ± SEM in μmol amino acid/g wet weight of gill tissue. (*n*), Number of replicates; MSX, methionine sulfoximine.

[a] Significant differences ($P < 0.05$) between clams exposed to sulfide (+Sulfide) and not exposed (−Sulfide).

[b] Significant differences ($P < 0.05$) between clams treated with inhibitor and not treated.

inhibitor of bacterial protein synthesis (Burnap and Trench, 1989), at a concentration previously determined to disrupt symbiont metabolism but to be nontoxic to the host (R. W. Lee, unpubl.). To examine the role of the host, clams were exposed to cycloheximide, a specific inhibitor of eukaryotic protein synthesis (Burnap and Trench, 1989), at a concentration nontoxic to the host for the duration of the treatment. In additional experiments, the effects of the ammonia assimilation inhibitor, methionine sulfoximine (MSX; Rees, 1987) were examined; MSX inhibits glutamine synthetase, which has been detected in *S. velum* tissues (Lee *et al.*, 1999). To ensure complete inhibition of ammonia assimilation, the MSX level was 10-fold higher than that utilized by Rees (1987). Taurine, hypotaurine, and thiotaurine levels in clams not exposed to sulfide were not affected by exposure to any of the inhibitors (Table 1). Additionally, the wet weights of whole clams were not altered by treatment with sulfide or metabolic inhibitors.

When clams were treated with the three metabolic inhibitors, the usual sulfide-induced increase in taurine levels was not observed (P values for comparisons between −sulfide and +sulfide, in the presence of inhibitors: chloramphenicol, $P = 0.552$; cycloheximide, $P = 0.451$; MSX, $P = 0.707$). Hypotaurine levels were not altered by sulfide exposure or treatment with inhibitors ($P = 0.064$). Thiotaurine levels increased after sulfide exposure, even in the presence of metabolic inhibitors (P values for comparisons between −sulfide and +sulfide in the presence of inhibitors: chloramphenicol, $P < 0.001$; cycloheximide, $P < 0.001$; MSX, $P < 0.0001$). This sulfide-stimulated increase, however, was reduced by treatment with chloramphenicol ($P = 0.016$, sulfide-exposed control clams *versus* chloramphenicol-treated sulfide-exposed clams).

Chloramphenicol and MSX decreased S. velum *sulfide and ammonia consumption*

The average rates of sulfide and oxygen consumption in the absence of inhibitors were 2.57 ± 0.06 (5) μmol · g^{-1} wet weight · h^{-1} [mean ± SE (n of experiments)] and 3.98 ± 0.01 (5) μmol · g^{-1} · h^{-1}, respectively. Chloramphenicol exposure completely halted sulfide consumption after 10 h (Fig. 2A, hours 120–130). Cycloheximide treatment did not affect sulfide consumption rates. Treatment with MSX inhibited ammonia uptake (Fig. 2B, hours 10–30).

Discussion

This study demonstrates that taurine and thiotaurine levels in *Solemya velum* gills increase after sulfide exposure. These two amino acids may function as nontoxic sulfide storage compounds. Inhibition of symbiont and host protein synthesis and host ammonia assimilation blocked sulfide-stimulated taurine synthesis; in contrast, only the inhibition

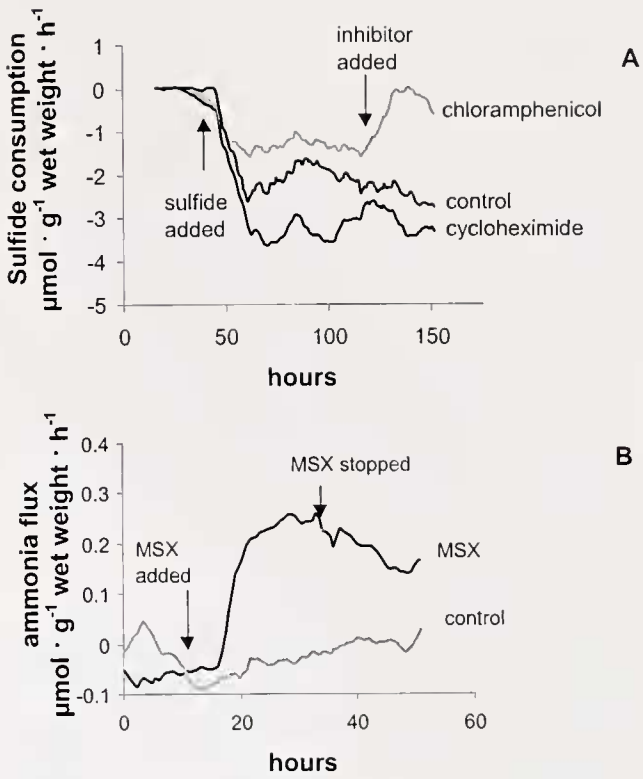

Figure 2. Effect of inhibitors on *Solemya velum* ammonia and sulfide fluxes. (A). Chloramphenicol (0.9 m*M*), an inhibitor of prokaryotic protein synthesis, blocked sulfide consumption. Cycloheximide (0.02 m*M*), an inhibitor of eukaryotic protein synthesis, had no effect. (B). Methionine sulfoximine (MSX, 0.25 m*M*), an inhibitor of ammonia assimilation, blocked ammonia uptake and resulted in ammonia excretion. Negative fluxes denote uptake from the medium.

of symbiont metabolism decreased the sulfide-stimulated thiotaurine synthesis. The maintenance of free amino acid pools depended upon the presence of functioning symbionts, sulfide consumption, and host ammonia assimilation. Thus, sulfur-containing free amino acids also may be a link between cycling of nitrogen and sulfur in chemoautotrophic symbioses.

The magnitude of changes in taurine and thiotaurine pools observed in the present study is sufficient for these amino acids to be physiologically significant sulfide storage compounds. In experiments in which clams were exposed to sulfide for 24 h, the increases in taurine and thiotaurine levels corresponded to synthesis rates of 0.89 μmol · g^{-1} wet weight · h^{-1} and 0.22 μmol · g^{-1} · h^{-1}, respectively. Since taurine contains one S atom and thiotaurine contains two S atoms, this corresponds to a potential sulfide incorporation rate of 1.33 μmol · g^{-1} · h^{-1}. The average rate of whole animal sulfide consumption measured under the control (no inhibitor) conditions was 2.57 μmol · g^{-1} · h^{-1}, which is similar to the sulfide consumption rate of *Solemya reidi* under similar experimental conditions (Anderson *et al.*, 1987). Therefore, the contribution of taurine and thio-

taurine to sulfide detoxification could account for up to 50% of the total sulfide flux.

Treatment with chloramphenicol, cycloheximide, and MSX prevented the sulfide-induced increases in taurine levels exhibited by the control clams. The lack of detectable taurine synthesis in chloramphenicol-treated clams likely can be attributed to the chloramphenicol-induced cessation of sulfide consumption. Additionally, chloramphenicol may act to prevent synthesis of mitochondrial proteins that are not nuclear encoded. Therefore, effects of chloramphenicol treatment might also be ascribed to impairment of mitochondrial metabolism. However, in preliminary experiments, *S. velum* tolerated treatment with 0.9 m*M* chloramphenicol for at least 9 days, suggesting that the effects due to chloramphenicol treatment are most likely the result of disrupted symbiont metabolism rather than toxicity to the host. Treatment with cycloheximide, which inhibits eukaryotic protein synthesis (Burnap and Trench, 1989) and is functionally analogous to chloramphenicol, decreased taurine synthesis in the presence of sulfide, but did not affect sulfide consumption. These results suggest that taurine is synthesized by the host and that the cycloheximide treatment did not affect any sulfide consumption which may occur in host tissues (Powell and Somero, 1986). Exposure to MSX blocked ammonia assimilation, probably contributing to the decreased taurine levels in MSX-treated clams. These results mirror those reported by Lee and coworkers (Lee *et al.*, 1997), who found a direct relationship between external ammonia availability and taurine levels in *S. reidi*.

Hypotaurine levels were not altered by exposure to sulfide or the metabolic inhibitors. These results suggest that hypotaurine is not directly involved in sulfide detoxification, nor is it an intermediate in the taurine synthesis pathway in *S. velum* (Cavallini *et al.*, 1976). Alternatively, hypotaurine could have protective functions, such as by serving as a compatible osmolyte (Yin *et al.*, 2000) or by scavenging free radicals (Huxtable, 1992).

Thiotaurine levels were greater in the gills of sulfide-exposed clams than in non-sulfide-exposed clams, regardless of exposure to metabolic inhibitors. Treatment with chloramphenicol reduced, but did not prevent, sulfide-induced thiotaurine synthesis. This reduction likely resulted from the chloramphenicol-induced cessation of sulfide consumption. Inhibition of host metabolic activity with cycloheximide did not affect thiotaurine levels in sulfide-exposed clams. Thiotaurine may be produced abiotically in host tissues, in which case host enzymatic pathways may not be necessary. Despite the MSX-induced inhibition of ammonia assimilation, thiotaurine levels in MSX-treated clams increased following sulfide exposure. Again, these results suggest that thiotaurine may be produced abiotically from precursors already present in the gill tissue and depend less upon ammonia availability.

Taurine and thiotaurine synthesis in S. velum

It is not known whether symbiotic bivalves maintain free amino acid pools by absorbing amino acids from the environment or by synthesizing them. We do know that *S. reidi* can take up free amino acids from sediment interstitial water (Lee *et al.*, 1992). However, sulfur-containing amino acids were not detected in the pore water samples from *S. reidi* burrows (Lee *et al.*, 1992) and were not present in the incubation medium in the experiments presented here, suggesting that solemyid clams synthesize taurine and thiotaurine. The biosynthesis pathways of taurine and thiotaurine in solemyid clams are unknown, but the results from this study suggest that these pathways require ammonia assimilation, sulfide consumption, and active symbiont metabolism.

Ammonia is present at elevated levels in the burrow environment of solemyid clams (Lee *et al.*, 1992; Krueger, 1996), and the clams assimilate it into amino acids, which may then serve as precursors for sulfur-containing amino acids (Lee and Childress, 1994). The present study indicates that glutamine synthetase is the primary enzyme in the assimilation pathway, since MSX treatment blocked ammonia uptake and caused ammonia excretion, similar to what was demonstrated in an algal-cnidarian symbiosis (Rees, 1987). The product of ammonia assimilation, glutamate, can then be used as a precursor in the production of taurine, which is a major product of ammonia assimilation in *S. reidi* tissues (Lee *et al.*, 1997; thiotaurine production was not tested for). Therefore, in *S. velum*, taurine and thiotaurine production in response to sulfide, as demonstrated in this study, may be facilitated by the ability of the bacterium-mollusc association to synthesize glutamate from inorganic nitrogen.

Just as glutamate likely contributes organic nitrogen to the synthesis of taurine and thiotaurine, the probable source of organic sulfur is cysteine. All of the demonstrated taurine synthesis pathways in mammalian and invertebrate tissues incorporate cysteine (Jacobsen and Smith, Jr., 1968; Bender, 1975; Bishop *et al.*, 1983; Huxtable, 1992), which apparently cannot be synthesized by molluscs (Bishop *et al.*, 1983). Although some intertidal molluscs utilize external cysteine sources to maintain taurine pools (Allen and Awapara, 1960; Jacobsen and Smith, Jr., 1968; Allen and Garrett, 1972; Bender, 1975), it is unlikely that solemyid clams take up cysteine from their environment (Lee *et al.*, 1992). Therefore, the most likely source of cysteine or other taurine precursors is the symbiotic bacteria. Gram-negative bacteria cannot synthesize hypotaurine, taurine, or thiotaurine (Jacobsen and Smith, Jr., 1968; Huxtable, 1992), but can make cysteine (Kredich, 1996). Cysteine synthesis in *E. coli* requires sulfur in the form of sulfide or thiosulfate (Stauffer, 1996) and assimilated ammonia in the form of glutamate (Reitzer, 1996). Translocation of the essential

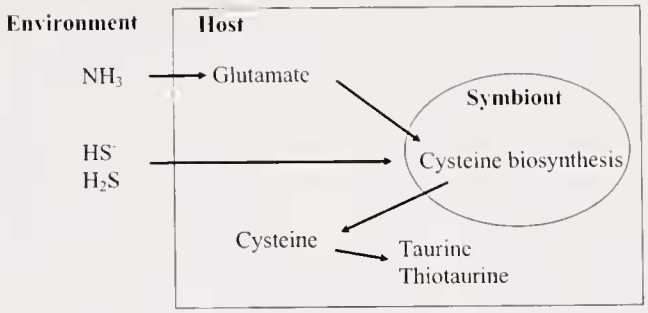

Figure 3. Proposed model of taurine and thiotaurine biosynthesis in *Solemya velum*. The clams extract ammonia and sulfides from the burrow environment. Ammonia is assimilated into glutamate, which is probably utilized by the symbionts in cysteine synthesis. Cysteine is translocated to the host and utilized in the synthesis of taurine and thiotaurine.

amino acid cysteine from gram-negative symbionts to host may occur in *S. velum* gill tissue as modeled in Figure 3. Such translocations have been demonstrated in bacteria-aphid and algal-cnidarian associations (Wang and Douglas, 1999; Douglas *et al.*, 2001).

The results of this study suggest that *S. velum* relies upon its symbionts as a source of taurine precursors, as modeled in Figure 3. The inhibition of symbiont metabolism with chloramphenicol, therefore, may equate to a loss of cysteine metabolism, thus decreasing sulfide consumption and taurine and thiotaurine synthesis by the host. Ammonia limitation, either by MSX treatment or reduced exogenous ammonia resources, may limit glutamate availability in *S. velum* tissues, thereby limiting cysteine production in the symbionts (Fig. 3). This could result in the lower taurine levels seen in the solemyid clams in this study and in previous work (Lee *et al.*, 1997). Thus, taurine and thiotaurine may be a link between nitrogen and sulfur cycling in chemoautotrophic symbioses and serve as nontoxic sulfide storage and transport compounds. The absence of similar patterns in nonsymbiotic sulfide-tolerant molluscs (*Geukensia demissa* and *Yoldia limatula*) suggests these functions of sulfur-containing free amino acids may be limited to symbiotic molluscs.

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